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INHIBITION OF QUORUM SENSING IN *Chromobacterium violaceum* CV026 BY VIOLACEIN PRODUCED BY *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa

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ABSTRACT

Quorum sensing (QS) regulates various activities of bacteria such as biofilm forming, virulence factors, swarming, and pigment production. Bacterium *Chromobacterium violaceum* produced violacein has also been regulated by quorum sensing system. The aim of this study was to evaluate the QS inhibition activity *buviolaceum* produced by the extract of *Pseudomonas aeruginosa* isolated from root of *Vetiveria zizanioides*. *P. aeruginosa* cultured on King's B agar, then was extracted using ethyl acetate as solvent. The extract was used to test anti-QS properties on *C. violaceum* CV026 at different concentration viz 0.0 mg/mL (control), 2.5 mg/mL, 3.0 mg/mL and 3.5 mg/mL. Violacein content of culture was measured by a spectrophotometer at a wavelength of 585 nm. The extract at 2.5, 3.0, and 3.5 mg/mL concentration had a significant effect on the reduction of violacein production by 31.6 %, 35.8 % and 70.3 %, respectively. While using an extract at the same concentration level there was no negative effect on the number of *C. violaceum* CV026 cells found. The results of study suggest that among the various tested concentrations, 3.5 mg/mL extract inhibits QS in *C. violaceum* CV026 via violacein production. Thus, the extract of *P. aeruginosa* has a very high potential to develop anti-QS.

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1 Introduction

Quorum sensing (QS) is a cell to cell communication mechanism that allows bacteria to control gene expression in order to respond to the cell density. Cell density can stimulate synthesis of small molecules (autoinducers) and QS regulates many activities such as bioluminescence, biofilm formation, virulence factor, and swarming (Defoirt et al., 2013). Anti-QS is a phenomenon of cell-cell communication related to molecules as intermediate. Molecules as an anti-QS agent could replace an inducer molecule which is responsible for the induction of protein expression to produce a vilurent agent. These anti-QS molecules can be used as a medicine.

C. violaceum is Gram-negative bacteria found in nature and that causes infections in human and animals. This bacterium can cause septicemia and abscess in lungs and in the liver (Petrilo et al., 1984). QS in *C. violaceum* regulates violacein piement production, antibiotic, hydrogen cyanide and some enzymes. *C. violaceum* has many genes involved in violacein production such as vioD, vioC, vioA genes which is arranged in an operon system mediated by N-Acyl Homoserine Lactone (AHL) (August et al., 2000). QS plays an important role in determining virulence factors and it has lead scientists to find a new target in developing treatment for disease caused by bacterial infections (Vasari et al., 2013). Actinobacteria, Firmicutes, Cyanobacteria, Bacteroidetes and Proteobacteria produce anti-QS enzymes (Kalia, 2013). Noncognate AHLs as intermadiates of AHL biosynthetic pathway, a dicyclic peptides, produced by bacteria as anti-QS or Quorum Quenching (QQ) molecules (Bauer & Robinson, 2002).

Medicinal plants have some endophytic bacteria which produces secondary metabolites which work as antimicrobial agents (Strobel, 2003). Nowadays, endophytes are a source for novel natural products in modern medicine, industry and agriculture (Yu et al., 2010). Most novel natural products possesing antimicrobial activities have been isolated from endophytes.

Endophytic microbes have been isolated from various plant tissues such as roots, leaves and stems. Fitriani et al. (2013) have isolated and characterized 17 isolates from the root of *Vetiveria zizanioides* on Luria Bertani agar media. Among these 5 isolates have been identified with *polyketide synthase* gene by polymerase chain reaction analysis. Based on *16S rRNA* analysis, one of them is *Pseudomonas aeruginosa* (Fitriani et al., 2013). *P. aeruginosa* is Gram-negative, aerobic and rod shaped bacteria belonging to the family Pseudomonadaceae. Further, Allu et al. (2014) isolated and characterized endophytic *P. aeruginosa* from red fruit pepper. Antimicrobial properties of the isolated bacterial strain were characterized against fungal pathogen *Colletotrichum*. Based on the study, biocidal properties of the *P. aeruginosa* were established and it was also suggested that isolated bacterium could grow in an artificial medium. The aim of this study was to evaluate the OS inhibition activities in term of violaceum production inhibition by the extract of *P. aeruginosa* isolated from root of *V. zizanioides*.

2 Materials and Methods

2.1 Preparation of bacterial culture

C. violaceum CV026 was obtained from Research Centre Microbial Diversity, Bogor Agricultural University, Indonesia, while the *P. aeruginosa* was isolated from the root of *V. zizanioides*. Isolated *P. aeruginosa* was maintained on the King's B agar (Pepton 2.0%; KH_2PO_4 0.15%; $MgSO_4$ 0.15%; glycerol (85%) 1.5% (v/v) and 2.0% Bacto agar (Difco, Spark, USA) at 37°C. The culture was rejuvenated after every two weeks. While *C. violaceum* CV026 was cultured in Luria Bertani (LB) agar and incubated at 27°C. Culture was rejuvenated for the interval of every 4 days. Before the treatment, culture was inoculated to LB broth and incubated at 27°C for 18-24 h in water bath and shaker.

2.2 Extraction of Endophytic *P. aeruginosa*

Extraction of *P. aeruginosa* was carried out by the method described by Niyaz Ahamed (2012) with some modification. Briefly, *P. aeruginosa* were cultured in 10 mL of King's B agar (Pepton 2.0%; KH_2PO_4 0.15%; $MgSO_4$ 0.15%); glycerol (85.0%) 1.5% (v/v); Bacto agar 2.0% (Difco, Spark, USA) broth with 37°C in temperature and 120 rev/min of shaking for 24 hours. The overnight culture was then inoculated into 90 mL of the same medium and condition. After their stationary phase, cultures were moved into centrifuge tube and centrifuged at 10000 rev/min for 10 minutes. Supernatants were then moved into separating flask and added by ethyl acetate (Merck, New York, USA) (1:1 v/v). Separating tube was hand-shaken constantly for about 15 minutes and left for 20 minutes. Then the upper middle layer that formed in the contained organic matter was taken. Extract was concentrated by vacuum evaporator with 50°C in temperature.

2.3 Anti-QS assay against *C. violaceum* CV026

Analysis of the anti-QS assay of *P. aeruginosa* extract was conducted as reported by Krishnan et al. (2012) with modification. One colony of *C. violaceum* CV026 was inoculated in a 50 mL LB broth medium and incubated for 18-24 h at 27°C with 110 rev/min agitations. Four mL of culture (OD_{600}=1.2) mixed with 20 mL LB (10.0 g/L Triptone, 5.0 g/L Yeast Extract, 5.0 g/L NaCl) (Difco, Spark, USA) molted agar. N-hexanoyl-L-homoserine-lactone (C6-HSL) (Sigma, St. Louis, USA) dissolved in DMSO 100%, was also added to agar with 1.2 µg/mL final concentration. The agar was mixed and poured into a Petri dish and wells were made in the center of the solidified agar plate. Three replicates were then made. A 40 µL of *P. aeruginosa* extract at 0.0, 2.5, 3.0, 3.5 mg/mL concentration was put into the well, respectively. The plates were kept in the incubator for 18-24 h at 27 °C and checked for inhibition of the violacein.

Table 1 Culture composition for each flask related violacein quantification.

Treatment	Culture composition	Volume	Final Concentration	Total Volume
Control +	LB medium	1998 mL	-	2 mL
	CV026 (OD_{600} 0.1)	1.1857 mL	1 x 10^8 CFU/mL	
	HSL (1 mg/mL)	2.4 µL	0.12 µL/mL	
	DMSO 1%	140 µL	0.07 % (v/v)	
Control -	LB Medium	1998 mL	-	2 mL
	CV026 (OD_{600} 0.1)	1.860 mL	1 x 10^8 CFU/mL	
	DMSO 1%	140 µL	0.07% (v/v)	
Extract	LB Medium	1.998 mL	-	2 mL
	CV026 (OD_{600} 0.1)	1.857 mL	1 x 10^8 CFU/mL	
	HSL (1 mg/mL)	2.4 µL	0.12 µL/mL	
	Extract1	140 µL	0.0; 2.5; 3.0; 3.5 mg/mL	

2.4 Violacein Quantification

Violacein production was analyzed by using spectrophotometry as described by Choo et al. (2005). Table 1 showed composition medium for each flask. One mL culture from each flask was centrifuged at 13000 rev/min for 5 min. The supernatant was discarded while the pellet was washed 2 times using buffer phosphat. One mL DMSO was mixed with the pellet and vortexed vigorously for 30s until the violacein dissolved completely. The cultures were then centrifuged at 13000 rev/min for 10 min. The absorbance of supernatant was read using the spectrophotometer (585 nm) to measure of violacein concentration.

2.5 Analysis of *C. violaceum* CV026 Cell Viability

The viability of *C. violaceum* CV026 cell was assessed by a total plate count (TPC). One hundred mL of *C. violacein* CV026 culture with *P. aeruginosa* extract was centrifuged at 13000 rev/min for 10 min to remove remaining extract in the culture. Later on the pellets were washed 2 times in 100 mL $PO4^{-2}$ and excess of *P. aeruginosa* extract was discarded. The culture was added to 100 mL Muller Hinton Broth (MHB). One mL of diluted culture to factors of $10^{-1} - 10^{-8}$ and one mL of culture from factors $10^{-6} - 10^{-8}$ pour into Muller Hinton Agar (MHA). The plates were incubated at 27°C for 18-24 h. The total of viable bacteria was then counted (Choo et al., 2005).

2.6 Antibacterial Assay

An antimicrobial assay was analyzed against *C. violacein* CV026 using the total plate count (TPC) procedure. One mL culture was serially diluted to factors of $10^{-1} - 10^{-8}$ and pourn into an MHA medium. The mixture was then left to be solidified and incubated at 27°C for 18-24 hours. The total viable bacterial cell was then counted. To ensure this test, a paper disc diffusion assay was also taken. 100 µL overnight culture of *C. violaceum* CV026 (OD_{600}=0.1) was spread on MHA. Paper discs containing extracts with current concentrations (0.0, 2.5, 3.0, 3.5 mg/mL) were loaded onto the plate. Plates were incubated in 27°C for 24 hours. Zone of inhibition around the disc was then observed (Sasidharan et al., 2011).

3 Results and Discussion

Reduction in violacein productions in *C. violaceum* CV026 are concentration dependent and reduced with the increasing *P. aeruginosa* extract concentration. Highest concentration of *P. aeruginosa* extract (3.5mg/ml) resulting in lower production of violacein as shown in Figure 1. *P. aeruginosa* extract could have influenced the mechanism of QS in *C. violaceum* through decreasing violacein production. The extract could have also interfered autoinducer production or interferred with the expression of the gene.

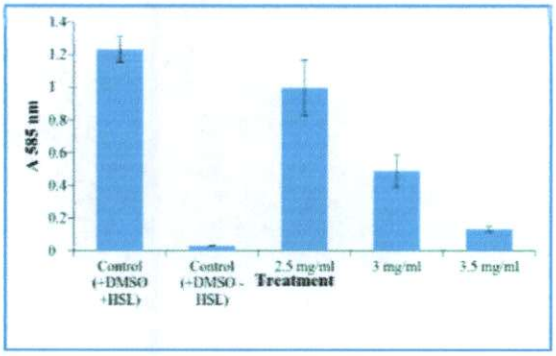

Figure 1 Decreasing violacein production by *P. aeruginosa* extract

Table 2 shows the result of quantification of violacein production in the presence of *P. aeruginosa* extract. Violacein production by cultures in the presence of *P. aeruginosa* extract was significantly different than the culture without *P. aeruginosa* extract (control). Highest inhibition in the violacein production was reported at the highest concentration (3.5 mg/mL).

Table 2 Decreasing Total Violacein production by Pseudomonas aeruginosa Extract using Spectrophotometer.

	Absorbance $_{595}$ ± SD		
	2.5 mg/mL	3.0 mg/mL	3.5 mg/mL
E+CV+C6-HSL	0.995 ± 0.17[b]	0.488 ± 0.1[c]	0.132 ± 0.019[d]
Control (CV+C6-HSL)	1.23 ± 0.08[a]		
Control (CV- C6-HSL)	0.0285 ± 0.002[e]		

Statistical analysis using Mann-Whitney U test; Different superscripts shows a significant difference ($P \leq 0.05$); E:Extract; CV: *Chromobacterium violaceum*; HSL: Homo Serine Lactone

According to Kalia & Pirohit (2011) violacein production might have reduced because of the activity of gene producing QS signal which can either inhibit or reduce violacein production. They also reported that structure of the signal was disrupted and the receptor sites with antagonist signal analogues were blocked. Heulier et al. (2006) suggested that *P. aeruginosa* has N-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-homoserine lactone (C4-HSL). They regulate the expression of a lot of genes in accordance with the induction of the transcription of *las*I and *rhl*I, as autoinducers.

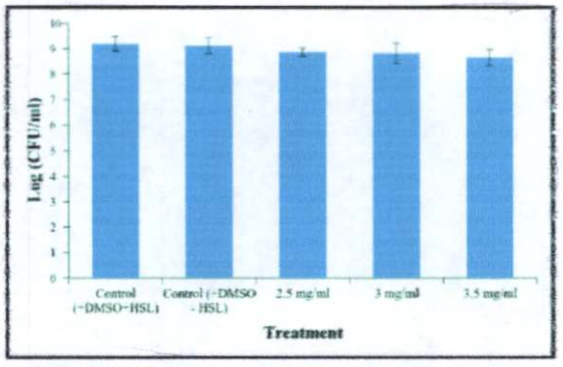

Figure 2 Viable cell of *C. violaceum* CV026 in the presence of *P. aeruginosa* extract

Table 3 shows total population of *C. violaceum* (log CFU/mL) in culture with extracts and controls. Total *C. violaceum* CV026 population did not show any significant difference in media with addition 0.0, 2.5, 3.0 and 3.5 mg/ml. Results of the study reveales that *P. aeruginosa* extract in media did not influence the viable cells and that the extract did not cause cell death.

The comparison of total bacteria also can be observed from figure 2. The decreasing violacein was not followed by decreasing of total viable bacteria. Meanwhile, antibacterial assay which carried by disc diffusion method, showed a similar result that *P. aeruginosa* had no inhibitory activity against *C. violaceum* CV026. The result of these assays indicates that extract of *P. aeruginosa* has potency as anti-QS in *C. violaceum*.

GC-MS analysis had been carried and revealed that the extracts of *P. aeruginosa* have potential as anti-QS compounds. According to Pratiwi (2013) *P. aeruginosa* extract contains 3-(1- phenyl - 2,3dihydro - 1H - isoindol - 2 -yl) propan-1-ol and 1H-Isoindole-1,3(2H)-dithione. Both compounds are indole derivatives and similar type of indole derivatives had been isolated from *Escherichia coli* by Li & Young (2013) and established anti-QS activity against *C. violaceum*. Similarly, Romano et al. (2014) reported the mechanism of this compound in inhibiting quorum sensing by inhibiting the activity of *vioA* gene which is one of many genes contained in *vioABCD* operon that is very crucial for violacein production in *C. violaceum*. Similar research showed that endophytic fungus *Penicillium* isolated from the stem of the milk thistle (*Sylibum marianum*) produces polyhydroxy anthraquinones as quorum sensing inhibitor (Figueroa et al., 2014).

Table 3 Antimicrobial Assay Against *C. violaceum* CV026

	Total viable bacteria (Log CFU/mL ± SD)		
	2.5 mg/mL	3.0 mg/mL	3.5 mg/mL
E+CV026+C6-HSL	8.86 ± 0.16[a]	8.81 ± 0.41[a]	8.65 ± 0.32[a]
Control (C6-HSL)	9.18 ± 0.028[a]		
Control (-C6-HSL)	9.11 ± 0.073[a]		

Statistical analysis by Duncan test; Different superscripts shows a significant difference ($P \leq 0.05$); E - Extract; CV: *Chromobacter violaceum*; HSL: Homo Serine Lactone

Overall, this study shows that *P. aeruginosa* isolated from the root of *V. zizanioides* produces secondary metabolites which has anti-QS properties. Furthermore, *P. aeruginosa* extract could inhibit violacein production in *C. violaceum* culture without killing the cell. Further research will purify the extract to get a single compound and be analyzed as an anti-QS compound.

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Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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